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Phenotypic and genotypic characterization of extended-spectrum β-lactamase genes in *Pseudomonas aeruginosa* isolates from local dogs bred in Nsukka, Nigeria

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Abstract

One of the major public health concerns is the transmission of antibiotic-resistant bacteria including extended spectrum beta-lactamase (ESBL)-producing Pseudomonas aeruginosa (P. aeruginosa) from animals to humans. This study investigated the prevalence of blaSHV, blaCTX-M, blaOXA and blaTEM ESBL genes in P. aeruginosa isolates from Nigeria local dogs bred in Nsukka. Anal swab samples (n = 150) were bacteriologically analyzed on soybean casein digest broth and cetrimide agar for the enrichment and isolation of P. aeruginosa respectively. Antibiotic sensitivity test was done according to Clinical and Laboratory Standard Institute (CLSI) criteria. Extended-Spectrum B-Lactamases production was detected phenotypically using the double disc synergy test (DDST) method and genotypically multiplex PCR technique respectively. A total of 39 (26.0 %) of the 150 samples were P. aeruginosa which were multiply resistant to antibiotics tested. The P. aeruginosa isolates were found to be multiply resistant to antibiotics tested. Twenty-One (53.9%) of the isolates showed ESBL phenotype while 18 (46.2 %) of the isolates were confirmed ESBL positive by Polymerase Chain Reaction (PCR). The prevalence of blaSHV, blaCTX-M, blaOXA and blaTEM genes in P. aeruginosa tested were 4 (10.3%), 17 (43.6 %), 15 (38.5%), and 6 (15.4%), respectively. This study, therefore, reported multi-drug resistant ESBL-positive P. aeruginosa in these local dog breeds. The exposure of these animals to antibiotics accumulates antibiotic resistance determinants in their intestinal flora.

Introduction

Pseudomonas aeruginosa (P. aeruginosa) is an opportunistic Gram-negative pathogen (1),

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colonizing diverse environments owing to its ability to dwell on a variety of organic food sources. The bacterium causes infection in some animals such as sheep, horses, bovines and dogs (2). In humans, it causes dermatitis, meningitis, skin infections in severe burns, sepsis, and nosocomial infections of the urinary tract (3, 4). This bacterium is also implicated in eye and digestive system infections (5). P. aeruginosa is reported as a common pathogen of both community (non-hospital) and hospital environments with the ability to form biofilms and resist antibiotics (6, 7). Thus, the bacterium is multidrug-resistant (MDR) in nature (8). Recently, the prevalence of MDR P. aeruginosa strains has been on the increase, with rates between 15% and 30% (9, 10). One of the major upshots of multidrug resistance is the difficulty of selecting an appropriate empirical antibiotic treatment. Patients with infections caused by MDR P. aeruginosa are prone to inadequate antibiotic treatment (11). When there is delay in receiving the right antibiotic therapy, the resultant effects include very high morbidity and mortality rates in patients with P. aeruginosa infections (12). The most common mechanism of resistance among the Gram-negative pathogens is the production of hydrolytic enzymes, the beta-lactamases. (13). Gram-negative bacteria that produce beta-lactamases are a major concern in healthcare due to their ability to spread globally. Complications in many bacterial infections are on the increase because of the increasing prevalence of beta-lactamases producing pathogens (14). The case is even worse with a class of enzymes called extended-spectrum beta-lactamases (ESBLs) which are enzymes that destroy most beta-lactam antibiotics, including cephalosporins and monobactams (15). The commonly encountered groups of these ESBL enzymes are TEM, SHV, OXA and CTX-M (15). The production of these enzymes is primarily plasmid encoded with multiple resistance genes including those of non-beta-lactam antibiotics (16-18), leaving clinicians with a limited choice of drugs in both human and veterinary medicine. Compared with human medicine, there is a paucity of information concerning ESBL-producing bacteria in veterinary medicine. The activity of these ESBL enzymes is inhibited by clavulanic acid, sulbactam, and tazobactam, and this phenomenon is utilized in the phenotypic detection of ESBLs. ESBL enzyme production is known in human clinical cases (19). Consequently, animals that are in close contact with humans contract ESBL-positive microorganisms in a reverse zoonotic event, called zooanthroponosis (20). Thus, food and companion animals are frequently implicated as potential reservoirs of multi-antibiotic-resistant pathogens (21). In Nsukka, Nigeria, local dogs serve as both companion and food animals. On a daily basis, these animals are slaughtered for business purposes just like any other farm animal. Their meats are consumed by the indigenes. So, humans are always in contact with the local dogs as either their companions or animals reared for their meat. The knowledge of the prevalence of P. aeruginosa in local dogs is important owing to its zoonotic and gene transfer potentials. The spread of these "high-risk" clones poses a threat to global public health that needs urgency and determination (21), thus, justifying the need for its study in the non-hospital environment to prevent any hazard that may be caused by them. In addition to monitoring the antimicrobial resistance patterns of P. aeruginosa isolates from both humans and animals, it is pertinent to elucidate the antibiotic resistance mechanism of these organisms to preserve global public health. Currently, there is no data on the genotypic characteristics of ESBL genes in P. aeruginosa from Nigerian local dogs in Nsukka. This is because adequate attention has not been given to the studies involving ESBL in P. aeruginosa isolates from local dogs in the area, possibly due to fear of the aggressive nature of some of these animals. Therefore, the objective of this study was to characterize, by phenotypic and genotypic methods, the ESBL genes among P. aeruginosa from Nigerian local dogs bred in Nsukka, South-East, Nigeria.

Materials and methods

Isolation and characterization of P. aeruginosa

One hundred and fifty (150) anal swab samples were used for the study and all the samples were analyzed using standard microbiology procedures. The samples were inoculated in soybean casein digest broth (Oxoid, UK) and incubated for 24 h at 37oC. Bacterial growth was suspected due to the turbidity observed after incubation. Each colony was sub-cultured on cetrimide selective agar (Oxoid, UK) for the selective isolation of P. aeruginosa (22). Suspension of broth culture showing turbidity was aseptically streaked onto cetrimide agar plates for the selective isolation of P. aeruginosa. The plates were incubated for 24 – 48 h at 37oC. Suspected P. aeruginosa isolates were further purified by culturing on cetrimide agar, the presence of which was confirmed by characteristic colonial morphology, Gram staining, growth at 42 ºC / 48 h in soybean casein digest broth and biochemical testing (23).

Phenotypic detection of the ESBL enzymes and antibiotic sensitivity testing

Inhibition diameter of less than 26 mm for both cefotaxime and ceftriaxone were selected for the Double Disc Synergy Test (DDST) for ESBL production by using a disc of amoxicillin-clavulanate (20/10 µg) along with two other antibiotics belonging to the third generation cephalosporins (3GC); cefotaxime (30 µg) and ceftriaxone (30 µg) (25) with some modifications. A liquid culture of each of all the bacteria to be analyzed (P. aeruginosa strains and the controls) was prepared by adjusting the prepared bacterial suspension to 0.5 McFarland opacity standard. Then, a lawn culture of the organisms was prepared by pouring the standardized liquid culture on a dried Mueller-Hinton agar plate and decanting the excess from the plate. An antibiotic disc of amoxicillin-clavulanic acid (20/10 µg) was placed at the center of the MH agar plate, and antibiotic discs containing cefotaxime (30 µg) and ceftazidime (30 µg) were each placed at a distance of 15mm (center to enter) from the central disc, amoxicillin-clavulanic acid 20/10 µg. The plates were incubated at 37oC for 18-24 h. Phenotypic inference for ESBL production was drawn from the expansion of the zones of inhibition of the cephalosporins by the amoxicillin-clavulanic acid disc (20/10 µg). A ≥5mm increase in the inhibition zone diameter for either of the cephalosporins (ceftazidime and cefotaxime) tested in combination with amoxicillin-clavulanic acid versus its zone when tested alone confirms ESBL production phenotypically. The positive and negative controls for ESBL production were Klebsiella pneumoniae 700603 and Escherichia coli 25922 respectively. The P. aeruginosa isolates were tested for their antibiotic susceptibilities by the disc diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (24).The following antibiotics were used; amoxicillin-clavulanic acid (20/10 µg)), meropenem (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), sulfamethoxazole-trimethoprim (25 µg), gentamicin (10 µg), ceftriaxone (30 µg), ciprofloxacin (10 µg), ofloxacin (10 µg), ampicillin (10 µg) and cloxacillin (10 µg). Other classes of antibiotics were used to test for multidrug resistance. All the antibiotics discs were sourced from Oxoid, UK. The diameter of the inhibition zone was measured and interpreted based on the CLSI criteria.

Screening for ESBL gene using PCR method

The phenotypically confirmed ESBL positive P. aeruginosa strains stored in slants were sub-cultured on nutrient agar plates and then on nutrient broth (Oxoid, UK) prior to DNA extraction. The pure single colony of the isolates was further subcultured in 5 ml of nutrient broth and incubated at 37ºC overnight for DNA extraction. Genomic DNA of the test isolate was prepared using ZR fungal/bacterial DNA miniprep (Zymo Research, California) following manufacturer's instructions. Primers used for amplification of resistance genes are shown in Table 1. ESBL-positive P. aeruginosa was examined for the presence of TEM, SHV, OXA-1 and CTX-M-1 genes by PCR technique. ESBL genes were amplified using a thermal cycler

(Lumex instruments, Canada) with a final volume of 26.5 µL master mix comprising 0.2 µL of Taq polymerase enzyme U/µL, 2.5 µl of 10X PCR buffer along with 2.5 µL MgCl2, 1 µL of 10 pM from each of the forward and reverse primers, 2.5 µL of dNTPs MIX (2 mM), 3 µL of DNA template (from the test isolates), 14.8 µL of nuclease-free water. Cycling conditions include initial denaturation at 94°C for 5 min, 36 cycles of denaturation at 94°C for 30 s and an initial elongation step at 72°C for 45 s, followed by the final elongation step at 72°C for 7 min.

Gel electrophoresis

Amplified PCR products were separated on 1.5 % agarose gel (3 g of the agarose powder/ 0.5X TBE buffers), according to standard procedure (25). The gel was run at 100 V for 1.5 h; and then visualized with UV transilluminator at 260 nm.

Statistical analysis

Data analysis was carried out using the Statistical Package for Social Sciences (SPSS) version 23.0 (SPSS, Chicago, IL, USA). Data were analyzed in terms of percentages.

Table 1: Primer sequence for ESBL gene characterization

Gene	Primer sequence	Expected amplicon size (bp)
*bla*TEM F	5'AAACGCTGGTGAAAGTA3'	500
*bla*TEM R	5'AGTGTGTTTAGAATGGTGATC3'	
*bla*OXA F	5'ACACAATACATATCAACTTCGC3'	650
*bla*OXA R	5'AGTGTGTTTAGAATGGTGATC3'	
*bla*CTX-M F	5'CGCTTTGCGATGTGCAG3'	750
*bla*CTX-M R	5'ACCGCGATATCGTTGGT3'	
*bla*SHV F	5'ATGCGTTATATTCGCCTGTG3'	850
*bla*SHV R	5'TGCTTTGTTATTCGGGCCAA3'	

Results

Prevalence of P. aeruginosa and ESBL genes

A total of 39 (26.0%) P. aeruginosa isolates were recovered from 150 anal swab samples used in the study. Isolates showed characteristic blue-green pigments which diffused into the medium, positive reaction to Oxidase test and growth at 42 ºC / 48 h in soybean casein digest broth. The presence of ESBL phenotypes in 21 (53.9%) out of the 39 isolates shows the appearance of ESBL positive and negative bacteria when cultivated on Mueller Hinton agar (MHA) in the presence of third generation cephalosporins (cefotaxime and ceftriaxone) and amoxicillin-clavulanic acid (Table 2).

Antibiotic susceptibility pattern of P. aeruginosa isolates and the prevalence of the encoding ESBL genes

The P. aeruginosa isolates exhibited different levels of sensitivity to the test antibiotics. The susceptibility patterns, as well as the ESBL genes encoding the resistance, are shown in Table 3.

Table 2: Prevalence of ESBL genes in the 39 isolates of *P. aeruginosa* from anal swab samples

Number of isolates n(%)	ESBL gene harboured
17(43.6 %)	*Bla*CTX-M
15(38.5%)	*Bla*OXA

6(15.4 %)	*Bla*TEM
4(10.3%)	*Bla*SHV

Table 3: Antibiotic susceptibility pattern of *P. aeruginosa* isolates and the distribution of the encoding ESBL genes (n = 39)

Antibiotics	**Susceptibility pattern**			**Encoding ESBL gene**
	Resistant	**n (%) Intermediate**	**Sensitive**	
Amoxicillin-clavulanic acid	22 (56.4)	8 (20.5)	9 (23.1)	*Bla*CTX-M,*bla*TEM,*bla*SHV
Meropenem	6 (15.3)	3(7.7)	30(77.0)	*Bla*OXA
Cefotaxime	20(51.3)	15(38.5)	4 (10.3)	*Bla*CTX-M,*bla*OXA
Ceftazidime	30 (77.0)	6 (15.3)	3(7.7)	*Bla*CTX-M,*bla*OXA
Sulfamethoxazole-Trimethoprim	27(69.2)	11(28.2)	1 (2.6)	*Bla*CTX-M,*bla*OXA,*bla*TEM, *bla*SHV
Gentamicin	20(51.3)	5(12.8)	14(35.9)	*Bla*CTX M,*bla*OXA,*bla*TEM, *bla*SHV
Ceftriaxone	20(51.3)	1(2.6)	18(46.0)	*Bla*CTX-M,*bla*OXA
Ciprofloxacin	20(51.3)	9(23.1)	10(25.6)	*Bla*CTX-M,*bla*TEM,*bla*SHV
Ofloxacin	20(51.3)	10(25.6)	9(23.1)	*Bla*CTX-M,*bla*OXA,*bla*TEM,*bla*SHV
Ampicillin	39 (100)	0 (0)	0 (0)	*Bla*TEM,*bla*SHV
Cloxacillin	38 (97.4)	1 (2.6)	0 (0)	*Bla*CTX-M,*bla*OXA,*bla*TEM,*bla*SHV

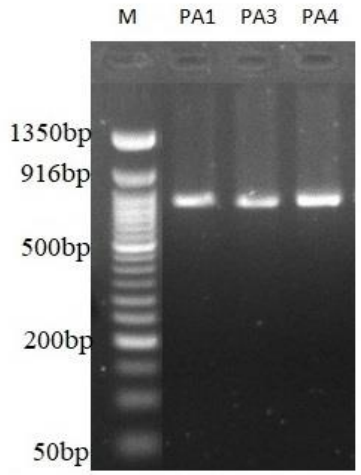

Fig. 1: Gel image of the amplified *bla* CTX-M gene showing amplification at 750bp. M is a 50bp ladder used for estimation of the size band of amplicons.

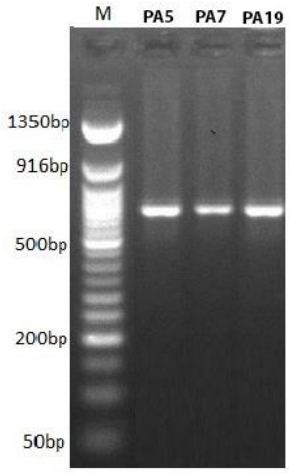

Fig. 2: Gel image of *bla* OXA gene showing amplification at about 650bp. M is a 50bp ladder used for estimation of the size band of amplicons.

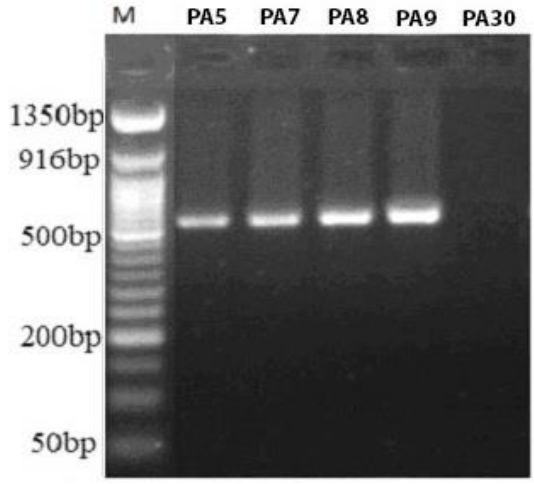

Fig. 3: Gel image of *bla* TEM gene showing amplification of TEM at about 500bp. M is a 50bp ladder used for estimation of the size band of amplicons.

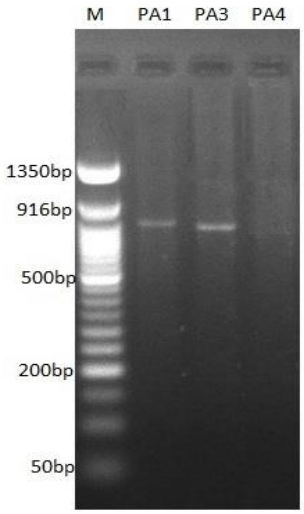

Fig. 4: Gel image of *bla* SHV gene showing amplification of SHV at about 850bp. M is a 50bp ladder used for estimation of the size band of amplicons.

Discussion

Antibiotics are important agents for the management of diseases caused by bacteria in both human and veterinary medicine (26). However, the increased emergence of antibiotic-resistant bacteria, including those that produce ESBLs have rendered many of these agents ineffective (13, 15). As a result, many of the multi-antibiotic resistant organisms remain viable even in the face of broad spectrum antibiotics (15). The need to search for the possible reservoirs of resistant organisms including ESBL producers from the non-hospital milieu ignited the zeal for this study. We, therefore, investigated the prevalence of ESBL phenotypes and genotypes; particularly blaSHV, blaCTX-M, blaOXA and bla-TEM genes in P. aeruginosa isolates from Nigeria local dogs bred in Nsukka. The isolation rate of P. aeruginosa in this study was high (n = 39, 26.0%), considering the nature and severity of diseases associated with this organism (Table 2). This result is not in agreement with the results of the work done by other researchers who isolated 16.7% and 17.7 % of P. aeruginosa strains from samples collected from healthy and sick dogs respectively (12). Similarly, several other reports of research carried out on dogs and other related animals revealed the presence of P.aeruginosa in these animals (27-29). In a related study conducted in Egypt (28), the prevalence of P. aeruginosa isolates in camel meat was about 22.5%, confirming the presence of this organism in other animals. With these results of the isolation rates of P. aeruginosa in samples from dogs, understanding the connections between P. aeruginosa and dogs is, therefore, very important for many reasons. Apart from helping veterinary doctors to choose the most effective antibiotic options for the treatment of sick dogs, understanding the transmission and prevention of P. aeruginosa infections in dogs can have implications for public health, especially as dogs can act as reservoirs of P. aeruginosa. In the present study, the antibiotic sensitivity test result of ESBL producing P. aeruginosa showed that these isolates were highly resistant to beta-lactam antibiotics in the penicillin family including ampicillin (100%), amoxicillin-clavulanic acid (56.4%) and cloxacillin (97.4%; Table 3) This is in agreement with the results of the work conducted in South Africa, which showed that most P. aeruginosa isolates from dogs were resistant to a wide range of antibiotics (30). A high proportion of

MDR P. aeruginosa isolates was resistant to Amoxicillin-Clavulanic acid (99%). This pattern was followed by resistance to antibiotics in the Cephalosporin family including Cefotaxime (51.3%), Ceftazidime (77.0%) and Ceftriaxone (51.3%). The reduced susceptibility of the isolates to the Cephalosporins as observed in the present work is similar to the results of the study conducted by (31, 29) in southwest Nigeria and Abakaliki respectively. Furthermore, Carbapenem class of beta-lactams represented by Meropenem showed 15.3 % resistance. A similar result showed that a low proportion of P. aeruginosa isolates were resistant to Imipenem (6%) (30). For non-beta lactam antibiotics, increased resistance of the isolates to fluoroquinolones (Ciprofloxacin and Ofloxacin) (51.3 %) and Gentamicin (51.3%) was recorded in this study, which is in agreement with the results of the same work conducted in South Africa (30). Conversely, a dissimilar result on the fluoroquinolone, cephalosporin and Gentamicin resistance profile of P. aeruginosa isolates from dogs was given in Japan because their findings showed lower resistance rates of Ciprofloxacin, Cefotaxime and Gentamicin which were 20.5%, 17.8% and 4.1%, respectively (32). Furthermore, such lower resistant rates to aminoglycosides and fluoroquinolones such as Ciprofloxacin (40.5%) have been reported in Malaysia (33) and India (34) respectively. Pseudomonas aeruginosa is remarkably resistant to antibiotics due to the outer membrane-associated permeability barrier of the organism (35). It is important to note that the addition of antibiotics in animal feed to combat infections and promote growth poses a health risk to the human population due to the possible development and dissemination of antibiotic resistance (36). Also in this study, ESBL production was phenotypically detected in 53.9% of all the P. aeruginosa isolates. This is similar to research conducted in Italy by some researchers who detected ESBL in P. aeruginosa isolates from dogs (37). In a related study carried out in Egypt (28), researchers reported a lower prevalence (45%) of phenotype of ESBL in isolates of P. aeruginosa from Camel. Unlike our findings, other researchers reported a much lower prevalence (20.8%) of ESBL producing clinical Enterobacteriaceae from dogs in Switzerland (38). The disparity in the prevalence of ESBL enzymes in these animals may be related to the differences in animal species, screening methods, dexterity of the researchers and the materials used for the tests. The PCR technique confirmed ESBL production in 18 (46.2%) of the P.aeruginosa isolates as shown in the gel images (Figures 1-4). The prevalence of blaCTX-M, blaOXA, blaTEM and blaSHV genes in P. aeruginosa tested were, 17 (43.6%), 15 (38.5%), 6 (15.4 %) and 4 (10.3 %), respectively. The presence of the blaCTX-M gene found in this study is in agreement with the result of the work done in Taiwan by other researchers who observed the blaCTX−M in 58.5% of the bla genes (39). Our findings on the prevalence of these ESBL genes are not in agreement with the report of the work done in India which showed that the predominant beta-lactamase genes detected in P. aeruginosa isolates from dogs include blaOXA (69.5%) followed by blaTEM (36.9%), bla-SHV (26.0%), blaCTX-M g (15.2%) and blaAmpC (4.34%) (40). In Egypt, it was observed that the most commonly detected β-lactamase-genes in P.aeruginosa were blaCTX-M (38%), followed by blaSHV (33.3%) and blaTEM (27). CTX-M enzymes have now been investigated to be the most disseminated type of ESBL globally and represent the wide dissemination of particular plasmids or bacterial clones (41). Both phenotypic detection and genotypic confirmation of ESBL genes in food and companion animals such as dogs is a grave challenge facing the human populace, especially in rural communities where hygiene and veterinary services are lacking.

Conclusion

We confirmed the notoriety of P. aeruginosa isolates from Nigeria local dogs for the production of ESBL enzymes. The high prevalence of the enzymes was corroborated by high prevalence of blaSHV, blaCTX-M, blaOXA and blaTEM genes in

the organisms isolated from the dogs. The presence of these genes has boosted the resistance of the organism to both beta-lactams and some non-beta-lactam antibiotics. The presence of ESBL genes in the isolates detected in this study is not far from several risk factors connected with the evolution and spread of antibiotic resistance genes in the non-hospital environment. It is possible that some of these isolates recovered from dogs were contracted from environments and/or owners and could be transmitted back to the owners and vice versa. The present study reporting the first detection of ESBL resistant phenotype and genotypes in P. aeruginosa strains of canine origin in Nsukka, South-East Nigeria consolidates our knowledge on the alarming ESBL resistance reports in the gut microbiota of dogs world-wide. The presence and persistence of these genes in non-human sources may pose a great risk to public health. Therefore, strict surveillance of this pathogen is very necessary to assuage the emergence and spread of multidrug-resistant strains, which can pose significant challenges in clinical settings.

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Ethics approval

The research was carried out following the World Medical Association (WMA) declaration of Helsinki on the principles for medical research involving human and animal subjects (42). There was no written ethical approval as the samples were collected at the point of slaughter.

Competing interests

The authors declare no competing interest.

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